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RESEARCH ARTICLE

Anticoagulant Difenacoum-induced Histological and Ultrastructurcal Alterations in Liver of Albino Rats

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Difenacoum is the second generation anticoagulant rodenticidehydroxycoumarin compounds (SGARs) that are more toxic compared to the first one. It is used as pesticides to control harmful rodents. The present work was piloted to evaluate the histological and ultrastructure effects after difenacoum inducement in the liver of albino rat. Oral administration of the two doses (0.25mg/kg and0.5 mg/kg) for two periods(2days and 4days) of difenacoum induced many histological and ultrastructure alterations in the hepatic tissue. The liver tissue showed congestion of blood vessels, leucocytic infiltrations, compressed sinusoids and enlargement of kupffer cells,necrotic nuclei and cytoplasmic vacuolization of fatty degeneration in the hepatocytes. Moreover, ultrastructure observations revealed shrinkage nuclei with condensation of heterochromatin and lipid droplets,destructedrough endoplasmic reticulum and mitochondria that were ill-differentiated cisternae.The observed alterations were dose and time dependent. Consequently, it is suggested that difenacoum induced vigorous hepatotoxicity concludedin the histological and ultrastructure changesof adult albino rat liver.The resulted severity was dose and time dependent.

Introduction

Anticoagulant rodenticides are the greatest ordinarily used pesticides to control harmful rodent populations (Albert et al., 2009).Difenacoum (4-hydroxycoumarin) the newest second generation anticoagulant rodenticide can lead to sub-lethal effects based on its half-life and toxicity data andhas the same mode of action as warfarin (Kerins, 1999; Prichard, 2013). It interference with vitamin K recycling by inhibiting of one or more of the enzymes, thereby preventing gamma-carboxylation of the precursors of blood clotting factors, II, VII, IX, and X.(Samama et al., 2002).

Rodenticides compounds commonly find their way to the environment through different ways. Therefore, it is obligatory to study in detail the possible impacts of these hazardous chemicals on the organisms. The second generation anticoagulant rodenticide are widely reported to contaminate and poison nontarget wildlife, primarily predator birds and mammals, and exposure pathways have not been well definite (Elliott et al., 2014) but their consumption accumulated the poison in the body (Fisher et al., 2003). Both domestic and wild animals are intoxicated by two principal routes: ingestion of anticoagulant-containing baits by accident (first option) or after eating dead rodents (second option) (Nikolov and Binev, 2008; Valchev et al., 2008). Furthermore, depending on their chemical structure, rodenticides are subdivided into first generation (couma-chlor, coumafuryl, coumatetralyl and warfarin) and second generation (brodifacoum, bromadiolone, difenacoum, difethialone and floco-umafen) compounds (Valchev et al., 2008).

Anticoagulant rodenticides inhibit vitamin K epoxide reductase resulting in a lack of active vitamin K. thereby inhibiting maturation of clotting factors that are not carboxylated and remain nonfunctional (Buckle, 1994;

Litovitz, et al., 1998; Murphy, 2002). Because anticoagulant rodenticides do not block these factors, their concentrations in blood decrease about 12-24 h after the intoxication coinciding with the first massive bleeding episodes (Smith et al. 2000; Petterino and Paolo, 2001; Kohn et al., 2003). Consequently, it was reported that hydroxycoumarin and indandione anticoagulant rodenticides yields toxic activity, clinical manifestations and hematological alterations or abnormalities (Buckle,1994; Samama et al.,2002).

The toxicity of difenacoum and other anticoagulant was studied in many animals. El-Daly (2002) reported that intoxications with difenacoumcause histological and ultrastructure alterations on rat kidney. Additional experimental intoxications (Valchev et al., 2009a; 2009b) with bromadiolone in dogs showed clinical and hematological alterations in this animal species. It was also shown that the intoxication was accompanied by hypothermia, tachycardia, polypnea, erythropaenia, reduced hematocrit, prolonged activated partial thromboplastin and prothrombin times, increased activities of aminotransferases (AST and ALT), blood glucose and total bilirubin. A number of kidney injuries are reported in human patients submitted to anticoagulant therapy with coumarin and its derivatives, renal failure, glomerulonephritis (Beauchamp et al., 2008; Brodsky et al., 2009;Kapoor and Bekali-Saab,2008; Remková et al., 2010). Furthermore, hyperemia, edema and hemorrhages in lungs (Mount, 1988; DuVall et al., 1989; Palmer et al., 1999, Binev et al., 2012). Battner et al. (2012) study the toxicity and potential risk of the anticoagulant rodenticide diphacinonein eastern screech- owles (*Megascopsasio*) and reported that single acute oral evoked coagulopathy and histopathological lesion (e.g. hemorrhage, hepatocellular vacuolation and or lethality at dose as low as 130 mg /kg b.w. and daily exposure to great quantity could result in liver mortality(Perevoshchikova, et al., 1978).

The increasing use of anticoagulant in a wide selections in wildlife making it an interesting subject to investigate its possible adverse effects on the liver as one of the main target organs for synthesis clotting factors. So, the aim of this study was to evaluate the histological and ultrastructure effects of difenacoum in liver of albino rats.

Material and Methods

The rodenticides:

Difenacoum is one of thesynthetic second generation rodenticide. Molecular Formula $C_{31}H_{24}O_3$. The ratacute oral toxicity (LD_{50}) is equal 1.8 mg/kg(Valchev et al., 2008)

Animals and experimental design:

In this experiment, Male albino rats (*Rattusnorvegicus*) obtained from Animal House in Zagazig Faculty of Veterinary Medicine, 3–4 months age weighing between 120 –140 g were used. They were housed in single wire mesh cages under standard conditions, with free access to the standard diet and water *ad libitum* and allowed to adjust to the new environment for two weeks before preliminaries the experiment in order to adapt themselves to their new environment and to ascertain their physical safety. The rats were housed at 25 ± 2^{o}C dark/light cycle. The experiment was performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 1996).Animals were randomly divided into five main experimental groups each of ten animals as follows:

1)Group I (control group): Animals were orally administrated with saline solution.

2)Group 2: Animals were orally administrated with 0.25mg/kg difenacoum (1/7 LD_{50}) dissolved in saline solution for 2 and 4days.

3)Group 3: Animals were given orally administrated with 0.5mg/ kg difenacoum (0.276 of LD_{50}) dissolved in saline solution for 2 and 4days.

At the end of the study all rats were decapitated. Liver pieces were collected to be subjected for assessment of histopathological examination using both light and electron microscopes.

For light microscopic study, the treated animals and their controls were sacrificed by decapitation. The livers were removed and fixed in 10% neutral formalin for 24 h. Fixed materials were processed and embedded in paraffin blocks. Sections of 5 µm thickness were cut. Slides were stained with haematoxylin and eosin described by Wilson and Gamble (2002) for histological examination.

For electron microscopic study, liver specimens were fixed in 2% glutaraldehyde and then post fixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Ultrathin sections were cut and double stained with uranyle acetate and lead citrate and examined by transmission electron microscope at electron microscope laboratory in Histology Department, Faculty of Medicine, Zagazig University (Glauret and Lewis, 1998).

Results

Histopathological examination:

Histological sections of control rat showed the normal hepatic cords of the parenchyma cells radiated form the central vein (CV). The hepatocytes contain one or two spherical prominent nuclei (N) and eosinophilic cytoplasm. The sinusoidal spaced arranged in-between the hepatic strand lined with fine endothelial cells and von Kupffer cells. No histopathological alteration was observed (Fig.1a).

Liver of rat treated with 0.25 mg/kg for 2days difenacoum displayed apparent signs of progressive changes compared to the control group. The normal structural organization of the hepatic lobules was impaired. Infiltration of inflammatory leucocytes was noticed around dilatation and congested central vein and other capillaries (Fig.1b). Impairment in the hepatocytes and necrosis signs with dense inflammatory cells and other debris was detected. Numerous pyknosis of the nuclei were visible (Fig.1c) in some cells. Vacoulation of hepatocytes cytoplasm as well as atrophy of the cells and clear infiltration of leucocytes in the sinusoids was observed. Dense collection of inflammation associated with congestion of liver central vein (Fig.1c) was observed. Moreover, kupffer cells in the hepatic sinusoids showed clear proliferated and hypertrophy.

Section in liver of rat treated with 0.25 mg/kg for 4days difenacoum demonstrated marked alterations than 2days, enlargement and congestion of blood capillaries, central and interlobular veins were noticed (Fig.1d). An obvious enlarged and proliferated bile canals were showed in occurrences of hyperplasia of bile ducts. Emigration of Leucocytes was visualized and paveminted the portal vein wall. The kupffer cell and endothelial cells were hypertrophy. Other figure of the same treatment showed a marked disorder of hepatic cords, enlargement and congestion of blood capillaries as well as atrophy of the parenchyma cells (Fig.1e). Inflammatory cells infiltration in different areas and in the sinusoids, vacuolated hepatocytes with pyknotic nuclei and the blood sinusoids appeared narrow or compressed and contained degenerated cells. Hemorrhage in the central vein was observed. Moreover, kupffer cells in the hepatic sinusoids were proliferated (Fig.1e).

H&E stained sections in liver of rat orally administration with difenacoum (0.5mg/kg for 2days) induced marked pathological changes in the hepatic cells, inflammatory leucocytic infiltrations and congestion of the intrahepatic blood vessels. Progressive degenerated hepatocytes accompanied by fragmented vacuolated and other karyolitic nuclei were detected (Fig.2a). Noxious hepatocytes necrosis and lysed cytoplasm with rupture of the cell membrane were recorded. Marked inflammatory leucocytic infiltrations all over the tissue were present. Other figure of the same treatment exhibit other lesions small karyolitic nuclei vacuolated cytoplasm besides narrowed sinusoids was detected (Fig.2b).

After increasing the time to 4days and the dose to 0.5 of difenacoum treatment, several features as degenerative hepatocytes were visible. The cells exhibited large number of cytoplasmic vacuoles and pyknosis of nuclei. Deeply blue basophilic cells of inflammatory infiltration inside the hepatic tissues were detected. The blood sinusoids could not be seen as they suffered from great narrowing (Fig.2c). Obvious severe degenerative hepatocytes represented by vacuoles of fatty degeneration in cytoplasm were exhibited in figure (2d). The same showed nuclei having deeply basophilic others were karyolitic nuclei. The hexagonal arrangement showed disarray of hepatic cords. Dark blue colour hematopoietic cells and hemorrhage are observed within the hepatic sinusoids and capillaries. Figure (2d) preserved the hepatocytes with more vacuolated and their nuclei suffered from pyknosis. The blood sinusoids became so narrow that they almost became obscure. Generally, orally administration of 0.5mg/kg for 4days difenacoum (1/7 of LC_{50}) induced more severe histopathological lesion in the liver compared with that of lower dose and short time.

Electron microscopy examination:

EM study of the liver of control rat cytoplasm contained plentiful organelles, predominantly mitochondria, smooth endoplasmic reticulum and rough endoplasmic reticulum. The hepatocytes membrane have gap junction between the two hepatocytes through the bile canaliculiexistent (Figs.3a).

EM investigation of rats treated with difenacoum (0.5mg/kg) for 4days showed that the liver cytological alterations were in harmony with those observed in H&E. The hepatocytes showed necrotic nuclei with irregular contour. Rarely clumped islets of heterochromatin fragmented through the karyolymph and around the thin nuclear membrane (Fig.3b). The cytoplasm contained clusters of rough endoplasmic reticulum and shrunken degenerated mitochondria with ill-differentiated cisternae with noticeable vacuoles in the cytoplasm (Figs. 3b). Moreover, Figure (3c) showed spherical nucleus with thin nuclear enveloped and free from the heterochromatin. The cytoplasm contains little vacuoles, swollen mitochondria, degenerated rER, some glycogen particles, ribosomes and microsomal fractions.

Besides, the same treatment (0.5mg/kg) caused destruction in the cell membrane containing gap junction through bile canaliculi between the two adjacent hepatocytes presents. The dilated bile canaliculi showed eradication of the microvilli-like projections. The cytoplasm appeared empty of cytoplasmic organelles. Less delimited mitochondria, vacuoles and some dense lysosomes were present (Figs.4a). Proliferated Von Kupffer cell associated macrophage was clear in narrow space of Disse with delimited precipitation of electron dense material and vacuoles

(Figs.4b). Lymphocytes appeared with rarefication cytoplasm and broken mitochondria. Furthermore, progress of numerous aggregated lipid droplets between destructed mitochondria indicative of severe fatty degeneration which were not evident in control group and dense bodies were detected inside the cytoplasm (Fig. 4c). Nucleus appeared with poorly chromatin material diffused through the karyolymph.

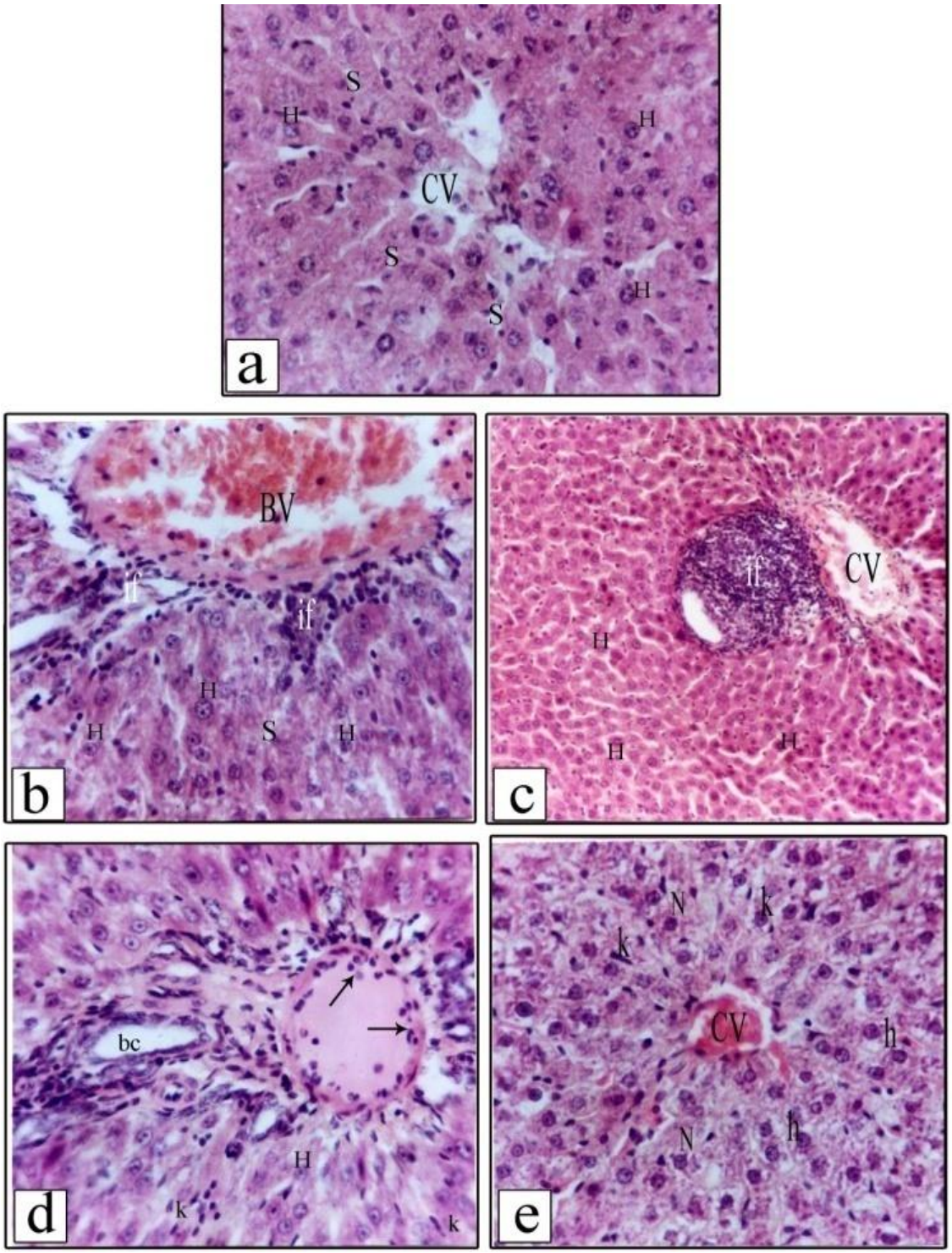

Fig.1(a). Section in liver of a control rat showing hepatocytes (H) radiating from the central vein (CV), continuation of sinusoidal spaces (S) lined with endothelial cells and von Kupffer cells (K),X400.**(b).**Liver of difenacoum treated rat (0.25mg/kg for 2days) showing degenerative changes in the hepatocytes (H). Dilatation

associated with hemorrhage in the central vein, inflammatory cells infiltration (if) in different areas and in sinusoids (S),X400.**(c).** Liver of a rat treated with difenacoum (0.25mg/kg for 2days) showing massive of inflammatory cells (if) along with the dilated the central vein (CV),X400.**(d).** Liver of a rat treated with difenacoum (0.25mg/kg for 4days) demonstrated a marked enlargement and congestion of portal vein as well as atrophy of the parenchyma cells. Leucocytes paveminted the portal vein wall (arrow), hyperplasia of bile canals (bc) and proliferated Kupffer cells (K),X400.**(e).** Liver of a rat treated with difenacoum (0.25mg/kg for 4days) displaying inflammatory cells infiltration in different area in the sinusoids, hypertrophy of kupffer (K) cells, some pyknotic nuclei (N) and congestion in the central veins (CV), disturbance in architecture of the hepatic lobule, X400.

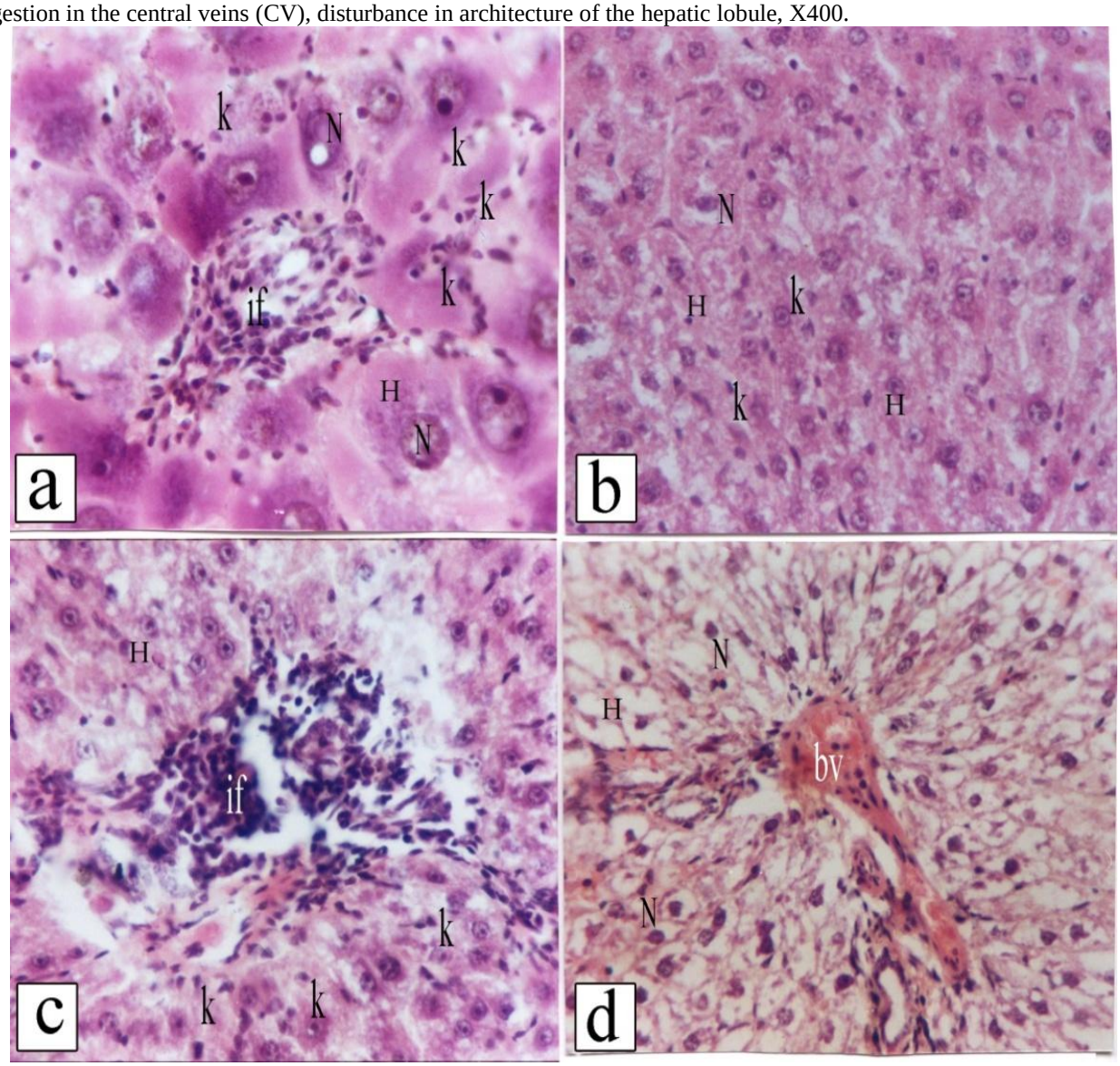

Fig.2(a). Section in liver of a rat treated with difenacoum (0.5mg/kg for 2days) revealed hepatic necrosis, karyolitic nucleus (N), inflammatory cells infiltrations (if) and activated kupffer (K) cells, X400.**(b).** Section in liver of rat treated with difenacoum (0.5mg/kg for 2days) revealed degeneration of the hepatocytes, compressed sinusoids and enlargement of kupffer cells (K), X400.**(c).** Section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) revealed impaired liver structure areas of inflammatory cells in different areas (if), X400.**(d).**Section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) showed parenchymal architectural collapse with hemorrhage (bv), severe cytoplasmic vacoulation and pyknotic nuclei (N), X400.

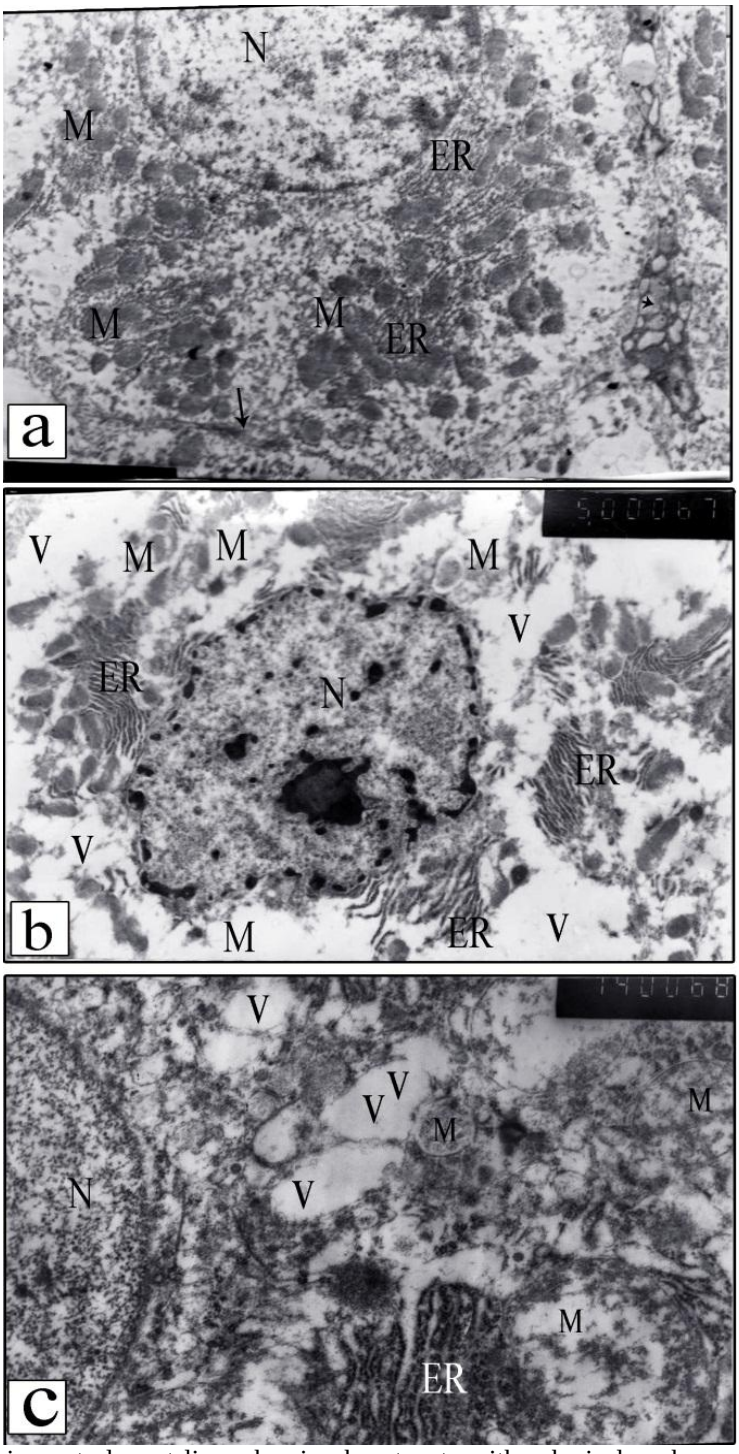

Fig.3(a).EM section in control a rat liver showing hepatocyte with spherical euchromatic nucleus (N). The cytoplasm contained parallel stacks of smooth and granular endoplasmic reticulum (ER), several mitochondria (M), gap junction (arrow), intercellular space (arrow head), X 4000. **(b).** EM section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) showing necrotic nucleus with poor clumped heterochromatin chromatin with irregular contour, the cytoplasm contained vesiculated rough endoplasmic reticulum(ER), and shrunken degenerated mitochondria(M) with ill-differentiated cisternae, large vacuoles (V), X 5000**, (c).** EM section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) showing nucleus with thin nuclear enveloped and free from the heterochromatin. The cytoplasm contains few vacuoles (V), swollen mitochondria, degeneratedER, fine granules, some microsomal fraction and firm glycogen particles, X 4000.

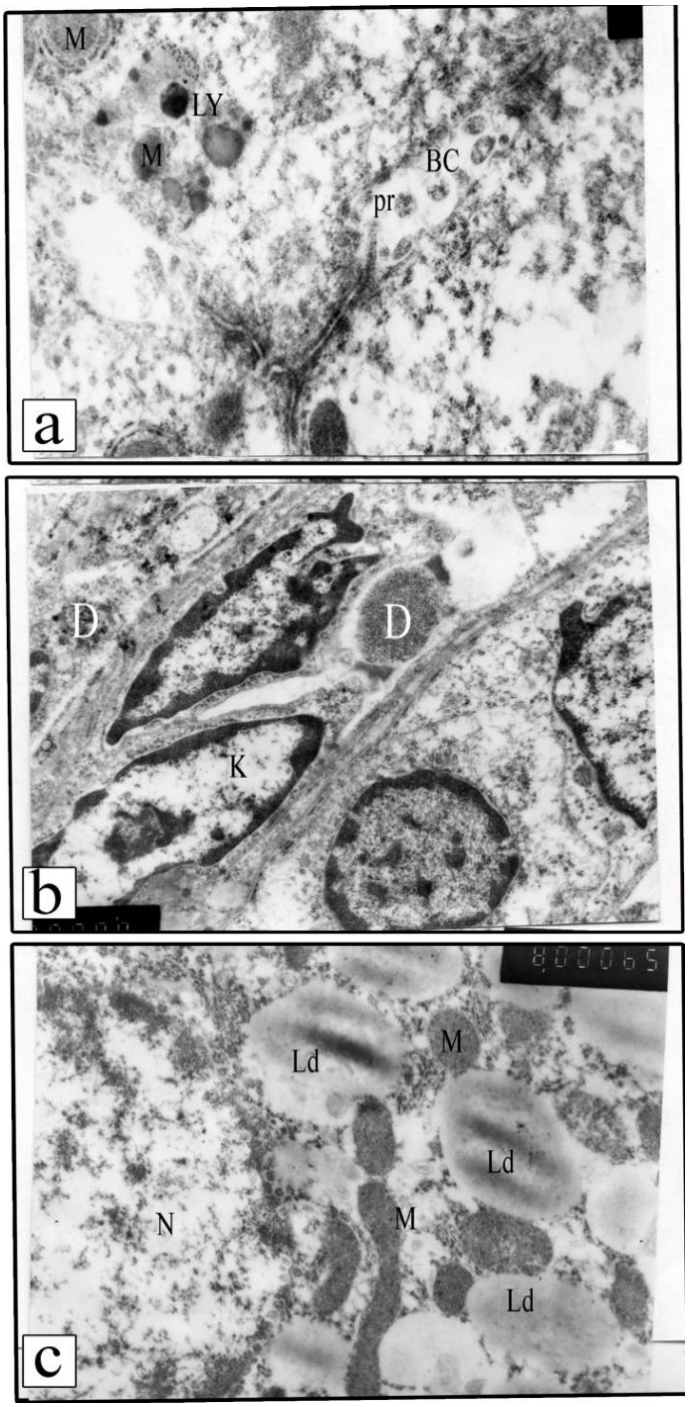

Fig.4(a). EM section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) demonstrated lateral intercellular space with dilated bile canaliculi (BC) between the two adjacent hepatocytes over eradication of the microvilli-like projections (pr), cell membrane containing gap junction, less delimited mitochondria(M), vacuoles and some dense lysosomes (LY) were present, X 17000.**(b).** EM section in liver of a rat treated with difenacoum (0.5mg/kg for 4days) demonstrated proliferated Von Kupffer cells(K) and macrophage were clear, besides narrow space of Disse confined precipitation of electron dense(D) material and vacuoles. Lymphocyte wasobserved and so rarefication cytoplasm, X 8000. **(c).** EM section in liver of rat treated with difenacoum (0.5mg/kg for 4days) showing progress of lipid droplets (Ld) between destructed mitochondria (M) indicative of drastic fatty degeneration. Nucleus (N) denoted poorly chromatin material diffused through the karyolymph, X 8000.

Discussion

In addition to their clinical use as oral anticoagulants, 4- hydroxycoumarins are applied world-wide as rodenticides. It is greatest benefit for medications as they prevent blood clots and serious complications as its potential hazardous effects occur from them.

Results obtained in this work showed that treating rats with difenacoun induced many histopathological changes in the liver. Similarly, El-Daly (2002) reported that difenacoun caused different histological and ultra-structural alterations in kidney of rat. The present histological effect of 0.25 and 0.5 mg/kg difenacoum treated rats liver displayed loss of the hepatic lobules architecture, infiltration of inflammatory cells and other debris in different zones of the hepatic lobules. The blood sinusoids suffered great narrowed, dilation of central and portal vein, congestion with lymphocytic infiltration and necrosis enlargement and proliferation of kupffer cells was recorded. These results are similar to those reported in different organs after treatment with second generation anticoagulants. Rattner et al. (2011), Rady et al. (2013) andBarnett et al., (2012) reported that bromadiolone and chlorophacinone anticoagulant induced hepatotoxicity in albino rats. Binev et al.(2011) reported that when Pheasants intoxication with the Bromadiolone; liver, kidney and lung showed strong hyperemia and activation of the monocytic-macrophageal system, granular parenchymal dystrophy, as well as necrobiotic to necrotic changes, intra- and inter-lobular hemorrhages, perivascular mononuclear proliferations and bile duct hyperplasia.

El-Banhawy et al. (1993) reported that exposure of rat liver to brodifenacoun caused blood congestion and mononuclear inflammatory cells infiltrations in the liver tissues. Moreover, administration of difenacoum to rats at a dietary dose of 0.2 mg/kg bw/day for 90days provided rise to clinical, hematological, biochemical and pathological findings indicative its toxic effects.

The observed hemorrhages, dilatation and congestion in the central vein and other capillaries after 0.25 and 0.5mg/kg difenacoum treated rats in the present study possible created as deficiency or suppression of the hepatic synthesis of vitamin K-dependent clotting factors. It may be attributed to the troubled coagulation and clotting mechanisms brought in the liver. Similarly, repeated oral administration doses of difenacoum to rats resulted in marked increase in clotting time and massive hemorrhaging in a wide range of tissues with deaths (Banett et al. 2012). Radi and Thompson (2004) reported that intoxication with accidental ingestion of brodifacoum, can cause prolonged bleeding. However, anticoagulant rodenticides inhibit vitamin K epoxide reductase following to lack of active vitamin K. This mechanism backs to blood clotting factors (II, VII, IX, and X) that are not carboxylated and remain nonfunctional (Buckle, 1994; Litovitz, et al., 1998; Murphy, 2002).

The present alterations in the liver after difenacoun treatment may explain by many factors; *firstly;* the coagulation flow involved oxidation reduction reaction tack place toward vit. K, a process contributed by release oxygen free radicals that may initiate series of damaged and unsafe reaction inside the cells. *Secondly;* calcium ions is likewise obligatory at other points in the coagulation cascade, in attendance of anticoagulant usually it existing in plentiful dangerous quantity inside the cells. *Thirdly;*phospholipid (a platelet membrane constituent) are required for the prothrombinase complexes to function and also anticoagulant difenacoum occurrence give vastly accumulation of the lipids in the cells. Previous report thatcalcium mediates the binding ability of the clotting factors via carboxylation of their glutamyl residues (Patocka et al., 2013). Vascular calcifications may induce by anticoagulant or vit. K deficiency(Krueger et al.,2009).

Electron microscope examination of rat treated with 0.5mg/kg difenacoum for 4days caused alteration in liver function by the indicative cell necrosis. Additional signs like shrinkage necrotic nuclei, condensation of heterochromatin and other nuclei free from the heterochromatin. The cytoplasm appeared with few of cytoplasmic organelles. The liver participates in synthesis of plasma proteins the present necrosis in the nuclei from difenacoum can be elucidated by the suppression of the hepatic synthesis to coagulation proteins resulting from decrease of vitamin K dependent clotting factors metabolism. Furthermore, Gill et al. (1991) discussed that the resistance to second generation anticoagulants involved by secondary mechanism that was under the control of several modified genes. Moreover, the same group of anticoagulants acts via inhibiting vitamin K recycling in the body then vit. K is needed for posttranslational modification of blood clotting proteins (FII, prothrombin; FVII, FIX, Christmas factor; FX, Stuart factor and proteins S and C (Brenner et al., 2009). They added that Vitamin K deficiency impairs normal blood clotting mechanism by preventing the carboxylation of essential glutamic acid residues in several blood-clotting proteins.

The present study showed severe swollen and damaged mitochondria after providing higher dose (0.5mg/kg) of the anticoagulant difenacoum. Anticoagulant may produce free reactive oxygen species due to alterations in the process of oxidation reduction reaction revealed by interrupting the cell turnover of vitamin K in liver. Other probability is the inhibition of the gene translation of many enzymes that participates in the coagulation process leading to destruction mitochondrial activity. Mignotte and Vayssiere (1998) revealed that many earlier studies disclosed that mitochondrial dysfunction contributed to apoptosis via the production of reactive oxygen species.

Also, it was reported that the major anticoagulant resistance gene (Greaves and Ayris, 1967) is thought to alter an enzyme or enzymes of vit. K cycle such that binding of and inhibition by anticoagulants is reduced (MacNicoll, 1993).The later author denoted that exists of specific genotypes are markers of warfarin resistance. Consequently, Park et al. (1986) suggested that intoxicated man with brodifenacoum and difenacoum either reserve capacity for vitamin KO reductase or an alternative mechanism for clotting factor synthesis inhibition. Parallel previous reports were recorded to the suppression of many enzymes after the anticoagulants. Arora and Goldhaber (2006) reported that anticoagulant-induced transaminase elevation is common and consider it as the initial marker of toxic liver. Inhibition of both phosphatases and nuclear RNase activity after heparin and spermidine was recorded by Perevoshchikova, et al. (1978). Additional biochemical abnormalities as Hypoproteinemia, hypoalbuminemia, hyperglycemia, bilirubinemia, increased urea concentration, and enhanced activities of alanine aminotransferase, alkaline phosphatase, and gamma glutamyltransferase (Binev et al., 2005; Kohn et al., 2003). Protein induced by vitamin K antagonism or absence (Crowther et al., 2000, and Spiller, 2003).

Ultrastructure examination in the present work showed that difenacoum caused rough endoplasmic reticulum to be shrinked and lipid vacuoles were abundant in the hepatocytes. The two elements were interrelated to each other hence the endoplasmic reticulum is responsible for lipids metabolism in hepatic cells. Lipolysis activity may be produced by alter neutral lipids absorption and assimilation. It was previously reported that difenacoum, brodifacoum are highly lipophilic, because the side chain has a high affinity for specific binding sites in tissues such as the liver (Hadler&Shadbolt, 1975; Park and Leck ,1982, Bachmann and Sullivan ,1983; MosterdandThijssen, 1991; Patocka et al., 2013). Furthermore,Pessayre et al. (2001) and Krahenbhul (2001) reported that inhibition of mitochondrial function together with accumulation of reactive oxygen species and lipid peroxidation and all these factors led to cell death. Moreover, the enzymes involved in the recycling of vitamin K reside in the rough endoplasmic reticulum of the hepatocyte (Olson, 1984; Suttie, 1987). Besides, additional factor may be the high lipophilicity of brodifacoum and other similar compounds preventing them from escaping from the binding compartment of the endoplasmatic reticulum. Benedetti (2005) stated that sER takes part in the synthesis of phospholipids for building of cell membrane and membranes of cell organelles. On the other hand, the increased rER was explained by important role in the synthesis and packaging of proteins (Kimball, 2008). Kurbegov et al.(2004) reported that coagulation factors II, VII, IX, X, and other vitamin K–dependent proteins require gamma-carboxylation of glutamic acid residues to be biologically active (to be able to bind calcium on the surface of platelet-derived phospholipid plugs and to participate in clotting reactions). From the present study it can conclude thatdifenacoum impaired the liver histology and ultrastructure and these changes were dose and duration dependent.

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